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Experimental Studies on the Etiology of Cholelithiasis

by

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INTRODUCTION

The etiology of cholelithiasis remains obscure though it is a common disease of mankind. The theory of NAUNYN (1892)[54] that gallstones are caused by inflammation and cholesterinization is usually taken as the starting point. THUDICHUM's[66], BOYSEN's[7], and ROVSING's[62] theories challenged his. They held that all or most stones are primarily pigment calculi formed in the intrahepatic canaliculi as a result of disease of the liver. In 1909, ASCHOFF and BACMEISTER[3] divided the cause of gallstones into "infective", "metabolic", and "static". Certain metabolic disorders[40], especially diabetes[27] and chronic alcoholism[19], were pointed out as having some correlation with the incidence of gallstones, but in most theories[39][61] on the cause of gallstones inflammation plays the major role.

In our laboratory, the results of studies on the physiological and surgical significance of essential fatty acids (EFA)[32][34][35] have suggested that alimentary factors may play the greatest role in gallstone formation. HIKASA[35] first pointed out in 1960 the possibility that gallstone formation might be due to deficiency and/or metabolic disturbances of EFA, and he and his collaborators demonstrated the various specific effects of EFA on cholesterol metabolism. HIRANO[36], YOSHINAGA[72], and MARUYAMA[44] observed that EFA and pyridoxine had a great influence on the synthesis of bile acids from cholesterol in the liver and their excretion into the bile in rats.

Attempts were made to produce gallstones in dogs[56], rabbits[4][6][67][70], guinea pigs[55], hamsters[14][64] and mice[65] by dietary means. Only in hamsters could cholesterol gallstones be produced experimentally by feeding them a fat- and cholesterol free diet. However, absolute EFA-deficiency was not observed even in patients with cholesterol gallstones[35]. Therefore, cholesterol gallstones must be produced even in hamsters fed a diet containing fat.

In the present studies, fats and pyridoxine, which might be assumed to be involved in EFA metabolism, are discussed as alimentary factors in gallstone formation, and the correlation between them and the development of experimental gallstones was examined in golden hamsters.

MATERIALS AND METHODS

Golden hamsters of both sexes weighing 30 to 55 g were used from our stock colony. Until the beginning of the experimental feeding period, the animals received a commercial diet, CE-2 (Central Laboratories of Experimental Animals, Tokyo). They were housed in individual screen-bottomed cages and were weighed weekly. Kneaded

synthetic food and water were supplied *ad libitum*. Diets containg fats were replaced every day to prevent oxidation of fatty acids.

These experiments consisted of the following four series :

Series I : On the effect of carbohydrate components.

Starch, glucose, sucrose, or fructose was used as the carbohydrate component. In each group, ten golden hamsters were fed on the fat- and cholesterol-free diet designed in our laboratory and described in Table 1.

Series II : On the effect of pyridoxine deficiency.

Golden hamsters were divided into six groups A to F as shown in Table 2. For protein, vitamin-free casein (Nutritional Biochemicals Co., OHIO, U. S. A.) was used, and pyridoxine was omitted from the vitamin mixture. Groups A, B, and C were fed starch as the carbohydrate component. As an antagonist to pyridoxine, desoxypyridoxine

Table 1 . Experimental Diets of Hamsters in Series I.

Group no.	A	B	C	D	CE-2
Starch	73.5%				
Sucrose		73.5			
Glucose			73 5		
Fructose				73.5	
Wheat & Corn					ca 60.
Fats					3.5
Crude casein	20.0	20.0	20.0	20.0	24.
Salt mixture‡	5.0	5.0	5.0	5.0	6.
Vitamins‡‡	1.0	1.0	1.0	1.0	
Choline Chloride	0.5	0.5	0.5	0.5	
Cellulose	—	—	—	—	1.5

‡ Salt Mixture

NaCl	4.6
NaH_2PO_4	9.2
K_2HPO_4	25.3
$CaH_4(PO_4)_2H_2O$	14.3
Ca lactate	36.9
Mg SO_4	7.0
KI	2.6
	100.0g

‡‡Vitamine Mixture (in 100g diet)

Vitamin	Our Laboratory	Dam et al.	CE-2
B_1	1 mg	5 mg	0.7mg
B_2	1.5mg	5 mg	1.0mg
B_6	1.0mg	5 mg	0.4mg
B_{12}	1 γ	—	2 γ
Folic acid	0.15mg	0.05mg	0.02mg
Niacin	10 mg	8 mg	8 mg
Biotin	—	0.05mg	—
C	37.5mg	5 mg	—
Ca pantothenate	2.5mg	5 mg	3.0mg
Inositol	—	15 mg	—
PABA	—	35.0mg	—
E	1 mg	5 mg	1.5mg
K	—	1.0mg	—
A	2500I.U.	(2000I.U.)	1000I.U.
D	200I.U.	(200I.U.)	200I.U.
Choline	(500mg)	200mg	140mg

(100γ/g of diet) was adminstered to Group B. ACTH-Z was injected in a dose of 1.5 units per day for four weeks in Group C. Group D received sucrose instead of starch. The other two sucrose fat-free diet groups (Group E and F) received agar-agar or C. M. C. (carboxyl methyl cellulose) as indigestible residues, which are considered to favor the growth of intestinal flora.

Table 2 : Experimental Diets of Hamsters in Series II.

Group no.	A, B, C	D	E	F
Starch	65.0%			
Sucrose		65.0	44.5	55.5
Sesame oil	5.0	5.0		
Lard	10.0	10.0		
Vitamin-free Casein	16.0	16.0	20.0	20.0
Salt Mixture	3.0	3.0	5.0	5.0
Pyridoxine-free Vitamins	0.5	0.5	1.0	1.0
Others	A : cholesterol 0.1%		Agar-agar 30.0%	C.M.C. 18.0%
	B : desoxypyridoxine 10mg/100g diet			
	C : ACTH-Z injection 1.5u./day			

Table 3 Fatty Acid Composition of the Dietary Fats.

Fatty Acid	Coconut Oil	Butter	Unsalted Butter	Lard	Sesame Oil	Cod Liver Oil
C 6 : 0,8 : 0	7.4	—	—	—	—	—
C 10 : 0	6.6	1.5	1.8	0.3	—	—
C 12 : 0	44.1	3.3	3.1	0.2	—	—
C 14 : 0	21.0	12.1	12.3	1.4	—	3.5
C 15 : 0	—	1.3	1.3	0.2	—	0.2
C 15 : 1	—	1.4	1.4	0.1	—	0.2
C 16 : 0	9.6	33.7	35.8	23.5	9.5	15.4
C 16 : 1	—	3.0	2.4	1.4	0.7	10.5
C 17 : 0	—	0.9	0.9	0.6	—	0.8
C 17 : 1	—	0.3	0.5	0.5	—	0.5
C 18 : 0	3.3	11.9	11.0	11.5	5.1	3.0
C 18 : 1	6.7	25.7	25.6	43.9	34.6	26.7
C 18 : 2	**1.5**	**2.5**	**1.8**	**10.8**	**48.9**	**4.2**
C 18 : 3	—	0.5	0.5	1.0	0.7	0.6
C 18 : 4	—	—	—	—	—	2.1
C 20 : 0	—	0.3	0.4	1.2	0.5	7.8
C 20 : 1 ?	—	—	—	0.4	—	0.6
C 20 : 3	—	—	—	0.2	—	—
C 20 : 4	—	—	—	—	—	—
C 20 : 5	—	trace	—	—	—	**15.1**
C 22 : 0or1	—	—	—	—	—	2.3
C 22 : 6	—	—	—	—	—	**9.8**

Series III : On the effect of EFA as fat component.

Five fats were used ; butter (canned by Snow Brand Milk Products Co.), coconut oil (Nakarai Chemicals Co.), lard (Prima lard made in U. S. A.), sesame oil (Takemoto Purified Oil Co.), and cod liver oil (Megane Kanyu Co.). As, in general, butter contains 2 % salt, unsalted butter (Snow Brand Milk Products Co.) and lard containing 2 % salt were also used in the experiments in this series. The fatty acid composition of these dietary fats was determined by gas-liquid chromatography (SHIMADZU Model GC-1B). The results are shown in Table 3.

The carbohydrate component in the diet containing sesame oil was starch, lactose or sucrose. In the other fat diets, glucose was used (Table 4).

Series IV : On the effect of fats and pyridoxal phosphate.

Pyridoxal phosphate (PALP), an active analogue of vitamin B_6, was injected subcutaneously in a dose of 40 mg/kg/day in the fat-free and four fat diet groups : butter, lard, sesame oil, and cod liver oil groups In addition, PALP was also injected in animals fed the diet containing pure linoleic acid (96 5 %) The carbohydrate component in the diet containing sesame oil was sucrose or glucose. In the other diets, glucose was used (Table 4).

Table 4 : Experimental Diets of Hamsters in Series III and IV.

Series III Group no.	A	B	C		D	E	F	G	H		
Series VI Group no.			B†	C	D			E	F	G	A
Starch	65.0%										
Lactose		63.5									
Sucrose			61.5								
Glucose				63.5	63.5	63.5	63.5	63.5	68.5	68.5	73.5
Sesame Oil	5.0	10.0	10.0	10.0							
Lard	10.0							10.0==			
Butter					10.0	10.0†††					
Coconut Oil							10.0				
Cod liver Oil									5.0		
Linoleic Acid										5.0	
Casein=	16.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0
Salt Mixture	3.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Vitamins ≡	0.5	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Choline Chloride	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
C. M. C.			2.0††								

= Vitamin-free casein was used in Series IV.
== Lard used in Series IV contained 2 % NaCl.
≡ Pyridoxine-free vitamin mixture was used in Series IV.
† The animals in Group A-G in Series IV were injected with PALP 1.5-2 mg/day.
†† C. M. C. was used as a prophylactic against diarrhea.
††† Unsalted butter was used in Group E instead of butter.

After the animals in these four series had been fed for 4-8 weeks, they were operated on under nembutal anesthesia. The common bile duct was ligated, the gallbladder removed, and a fine polyethylene tube was inserted into the hepatic duct through the

cystic duct. The hepatic bile was collected for 24 hours and analyzed as follows:

(1) Biliary cholesterol was measured according to LIEBERMANN-BURCHARD'S method modified by MARTENSSON (1963).[43]

(2) Bile acid concentration was calculated by an adaptation of the procedure reported by MOSBACH et al. (1954)[50].

(3) According to the method of FAWCETT et al. (1961)[24], calcium was determined by flame spectrophotometry.

(4) Phopsholipids in the bile were extracted by Bloor's solution and measured by the method of MORRISON (1964)[49].

(5) Total fatty acids were analyzed by gas liquid chromatography by the method of HIKASA et al. (1963)[34].

(6) Bile pigments (free, mono- and di-glucuronated bilirubin) were measured by the solvent-partition method of EBERLEIN (1960)[22].

After cholecystectomy, the gallstones in the bladder were collected and washed with distilled water and dried immediately. The stones were classified according to microscopic appearance, chemical analysis and infrared spectrometry (Japan Spectroscopic Manufacturing Co. DS-301).

RESULTS

Series I :

Cholesterol gallstones (Fig. 4) were very regularly produced in hamsters fed a fat-free diet with glucose as the carbohydrate component, but among those fed sucrose or fructose, the incidence of gallstones was about half that in the glucose group, while the animals fed starch produced no gallstones (Table 5).

The total bile acid concentration in the bile of those fed glucose or fructose was less than in that of the starch diet group. Conversely, the cholesterol level in the bile was higher in the glucose and fructose fed groups than in the starch fed group, and dried specimens of bladder bile from animals with cholesterol stones contained many cholesterol crystals (Fig. 6). And the ratio of total bile acids to cholesterol was much lower in the former groups than in the latter. The phospholipid level in the bile was higher in the glucose than in the starch group, but no difference was noted in the ratio of phospholipid to cholesterol (Table 6).

Series II :

The administration of 0.1% crystal-cholesterol or the injection of ACTH-Z in the pyridoxine-deficient starch diet group did not cause cholesterol gallstones to be formed, nor did the addition of desoxypyridoxine to the pyridoxine-deficient starch diet (Table 5). The animals in the desoxypyridoxine group had already been fed the pyridoxine-deficient diet for about two months, and the wall of the gallbladder was very thick. However, no bacteria were found in the gallbladder bile. Because its negative result of cultures with blood-agar, 101-medium for staphylococci, and Drigarsky's medium was obtained.

In the group fed a pyridoxine-deficient sucrose diet containing fat, only pigmented stones were produced (Fig. 5). These green or dark brown stones were very hard and contained an abundance of phosphorus and calcium and very small amount of biliverdin by chemical analysis. A moderate amount of bile salts was found by infrared spectrometry,

Table 5 : Incidence of Gallstones in Hamsters fed Various Diets.

Experimental Series & Groups		Diet characteristics	no. of animals	Initial weight (g)	Weight gain during 4 weeks (g)	Maximal feeding period (days)	Survivors after 4 weeks	Incidence of gallstones among survivors	
								Cholesterol stones	Pigmented stones
I	A	Starch fat-free	10	37.5	3.0	35	8	0%	0%
	B	Sucrose fat-free	10	37.4	6.0	56	7	43%	0%
	C	Glucose fat-free	10	51.1	−1.1	35	7	86%	0%
	D	Fructose fat-free	10	45.6	7.8	46	8	50%	0%
II	A	Starch fat* cholest.	14	48.9	29.8	63	14	0%	8%
	B	Starch fat* ACTH-inj.	10	48.9	− 5.5	28	10	0%	0%
	C	Starch fat* Desoxypyrid.	10	48.9	− 4.3	28	10	0%	0%
	D	Sucrose fat*	15	67.8	16.5	45	14	0%	43%
	E	Sucrose fat-free C. M. C.	10	47.1	12.5	43	9	11%	22%
	F	Sucrose fat-free Agar	10	44.4	0.3	42	9	22%	33%
III	A	Starch fat*	16	41.7	9.6	28	15	0%	0%
	B	Lactose sesame oil	10	44.0	2.0	36	8	0%	0%
	C	Sucrose sesame oil	10	43.8	17.5	36	8	0%	62%
	D	Glucose butter	10	49.1	5.5	43	8	75%	0%
	E	Glucose butter unsalted	10	53.1	21.9	34	10	90%	0%
	F	Glucose coconut oil	10	53.3	15.1	34	10	80%	10%
	G	Glucose lard	10	46.8	− 5.4	34	10	40%	0%
	H	Glucose cod liver oil	10	42.5	2.1	28	6	0%	0%
IV	A	Glucose fat-free PALP	10	52.2	7.8	28	6	83%	0%
	B	Sucrose sesame oil PALP	13	50.4	16.1	42	13	0%	23%
	C	Glucose sesame oil PALP	10	30.2	21.4	39	10	0%	0%
	D	Glucose butter PALP	10	41.8	16.0	35	9	78%	0%
	E	Glucose lard PALP	10	37.0	18.9	40	10	0%	0%
	F	Glucose cod liver oil PALP	10	43.1	18.5	35	9	0%	33%
	G	Glucose linoleic a. PALP	10	41.8	22.4	32	6	0%	0%

* 'fat' indicates 5% sesame oil and 10% lard.

Table 6 : Biochemical Analysis of the Bile of Hamsters fed Various Diets.

Experimental Series & Groups		Diet characteristics	Cholesterol (mg/dl)	Total bile acids (mg/dl)	Phospholipids (mg/dl)	Ratio of total bile acids to cholesterol	Ratio of Phospholipids to cholesterol
I	A	Starch fat-free	6.0±0.7	79.3±10.1	10.6±3.1	12.0±2.8	1.6±0.4
	C	Glucose fat-free	10.8±1.4	56.3±7.1	17.9±7.8	5.3±1.2	1.6±0.4
	D	Fructose fat-free	10.9±0.7	52.0±11.8	20.3±3.1	4.8±1.5	1.4±0.8
III	A	Starch fat	6.5±1.5	77.2±14.6	18.6±4.3	12.3±2.4	3.2±1.2
	D	Glucose butter	12.9±2.5	56.8±14.5	13.7±7.5	5.8±1.6	1.0±0.6
IV	B	Sucrose sesame oil PALP	3.5±1.8	73.6±10.5	18.1±6.4	24.3±7.7	5.5±1.7
	C	Glucose sesame oil PALP	7.7±2.1	85.7±7.1	31.8±18.1	12.1±2.7	4.4±1.4
	D	Glucose butter PALP	19.8±7.1	101.5±14.1	25.6±10.4	6.0±2.4	1.4±0.6
	E	Glucose lard PALP	12.8±4.6	85.1±12.2	20.1±11.3	8.1±3.1	1.7±0.9
	F	Glucose cod liver oil PALP	4.5±1.9	60.3±8.7	19.6±11.3	15.1±3.2	4.1±2.2

Experimental Series & Groups		Diet characteristics	Ca^{++} (mEq/L)	Total bilirubin (mg/dl)	Fractions of bilirubin (%) free-from	mono-glucuronade	di-glucuronade
I	A	Starch fat-free	5.6±0.2	4.9±0.1	34.3±3.0	38.5±5.2	27.2±2.2
	C	Glucose fas-free	5.4±0.2	5.6±1.3	34.6±6.3	43.4±4.4	21.9±5.2
	D	Fructose fat-free	5.0±0.5	4.5±0.2	34.2±3.3	45.0±6.0	20.8±2.6
III	A	Starch fat	5.9±0.3	5.7±1.4	26.1±2.2	40.3±6.8	33.6±6.0
	D	Glucose butter	5.7±0.6	4.6±0.6	35.1±6.5	50.4±3.0	14.5±3.4
IV	B	Sucrose sesame oil PALP	5.3±0.1	4.1±1.1	34.7±5.3	42.6±2.6	22.6±5.7
	C	Glucose sesame oil PALP		4.5±0.5	36.5±6.1	41.7±5.4	18.8±3.9
	D	Glucose butter PALP		5.2±0.5	41.7±1.2	41.2±1.2	14.0±3.0
	E	Glucose lard PALP		6.4±1.1	29.1±9.2	50.7±7.9	18.7±2.3
	F	Glucose cod liver oil PALP		1.9±0.6	37.1±4.2	47.8±4.7	15.0±4.3

Table 7 : Fatty Acid Composition of the Bile of Hamsters fed Various Diets.

Fatty acid	Starch fat-free	Glucose fat-free	Glucose coconut oil	Glucose butter	Glucose lard	Glucose sesame oil	Sucrose sesame oil	Glucose cod liver oil	Glucose linoleic acid	CE-2
<C 14 : 0	0.4	0.4	2.9	0.5	1.0	3.1	5.1	0.7	0.1	2.6
C 15 : 0	0.8	0.3	trace	0.4	0.7	2.7	1.2	0.3	0.1	1.7
C 16 : 0	33.9	33.3	25.8	31.6	31.9	34.5	31.7	41.8	17.5	35.1
C 16 : 1	5.9	6.3	1.2	4.9	3.5	3.2	3.8	5.1	1.3	1.7
C 17 : 0	1.1	0.8	2.1	0.8	0.4	trace	1.8	1.4	0.3	0.6
C 18 : 0	2.6	3.3	4.6	1.4	5.1	4.5	4.5	3.7	2.8	5.6
C 18 : 1	**48.0**	**46.7**	**47.0**	**46.3**	**38.5**	**20.9**	**17.4**	**32.6**	**5.8**	**18.3**
C 18 : 2	**3.9**	**3.6**	**3.9**	**5.7**	**12.3**	**24.8**	**23.9**	**3.3**	**68.7**	**30.6**
C 18 : 3	trace	0.5	trace	0.9	trace	—	—	—	0.8	0.4
C 20 : 3	**2.8**	**3.8**	**4.3**	**2.0**	**1.1**	—	—	—	—	
C 20 : 4	**0.7**	**0.5**	**2.1**	**1.5**	**2.3**	**6.0**	**3.2**	**1.6**	**2.2**	**2.4**
>C 20 : 5	—		—	—	—	1.5	—	6.2		1.1

Table 8 : Composition of Pigmented Stones in Hamsters.

	Color	Weight	Cholesterol	Biliverdin	P	Ca
1	dark brown	1.42mg	trace	3.1%	8.4%	11.1%
2	black	1.32mg	—	5.5%	7.5%	10.6%
3	brown	0.7 mg	trace	1.4%	15.8%	22.5%
4	green	6.55mg	trace	2.8%	11.5%	14.5%

and cholesterol was observed to be a trace element by these two methods (Table 8). The addition of C. M. C. or agar-agar as cellulose to the sucrose fat-free diet decreased and delayed the development of cholesterol gallstones (Table 5).

Hepatic bile was not collected in these groups in Series II.

Hamsters fed a starch diet excreted large yellow feces, which contained much indigested starch. They were also inspected through the intestinal wall at operation. The animals fed a sucrose diet with fat had rough fur which changed to a dark brown. Sometimes their feces were mud-colored and diarrheal. Rectal prolapse and anal bleeding were also frequent in the sucrose diet group.

Series III :

Starch diet containing 5 % sesame oil and 10 % lard as fat component completely prevented the formation of cholesterol gallstones, and the incidence of cholesterol gallstones in the group fed a lactose diet containing 10 % sesame oil was also none. In those fed a sucrose diet containing 10 % sesame oil, many pigmented gallstones developed, but no cholesterol stones. When cod liver oil was added to the glucose diet, no cholesterol gallstones formed.

However, a glucose diet containing 10 % butter fat did not prevent cholesterol gallstone formation in spite of the presence of considerable amounts of linoleic acid (about 200 mg/day). The animals grew normally and lived through the experimental period. These gallstones were slightly yellow and contained more than 80 % cholesterol by analysis (Fig. 7).

The use of "unsalted butter" instead of the usual butter containing 2 % salt did not affect the incidence of gallstones. Among animals fed the glucose diet with 10 % lard containg 2 % salt, cholesterol gallstones were observed in 40 % of the surviving animals.

In the glucose coconut oil diet group, cholesterol gallstones were produced regularly, but the weight gain of animals in this group was less in the butter diet group (Table 5).

The levels of total bile acids and cholesterol in the bile of hamsters fed the glucose butter diet were very similar to those in the glucose fat-free diet group with cholesterol gallstones, and the addition of fat to the starch diet caused an increase of phospholipids only (Table 6).

Series IV :

All the hamsters on the glucose fat diet which received simultaneous injections of PALP (pyridoxal phosphate), grew healthfully, but PALP did not prevent cholesterol gallstone formation in the glucose-butter and fat-free diet groups. PALP injection prevented the development of cholesterol gallstones in the hamsters fed glucose-lard and sucrose-sesame oil diets, but a few of these developed pigmented stones. No gallstones were fou

in the glucose sesame oil diet group treated with PALP injections, and the bile in their gallbladders was clear in all cases. Pigmented stones were found in three hamsters fed the cod liver oil glucose diet treated with PALP injections (Table 5).

The cholesterol level in the bile was highest in the glucose-butter group and lowest in the glucose-cod liver oil group. It was much lower in the sucrose-sesame oil group. Total bile acid concentration in the bile increased markedly in the butter, lard, and sesame oil groups treated with PALP injections. In the glucose-butter group, the ratio of total bile acids to cholesterol in the bile decreased abnormally. But, in the glucose-sesame oil and cod liver oil groups, the ratio was normal. The ratio was slightly decreased in the glucose-lard group. The phospholipid level in the bile was increased by PALP injections in all groups. The ratio of phospholipid to cholesterol, however, was the same only in the glucose-butter and -lard groups as in the fat-free groups (Table 6).

The composition of total fatty acids in the hepatic bile was of great interest (Table 7). In the fat-free glucose and starch diet groups in Series I, a marked increase of oleic (18:1) and trienoic (20:3) levels and a decrease of linoleic (18:2) and arachidonic acid (20:4) levels in the bile were observed in the gas-liquid chromatogram. This pattern was the same in the coconut oil, and butter groups. The levels in the lard group were intermediate between those in the butter and the sesame oil groups. In the cod liver oil group the bile contained several highly unsaturated fatty acids, a great amount of which are present in this oil. The addition of pure linoleic acid to the glucose diet caused only an abnormal increase of linoleic acid instead of a decrease of oleic acid.

The calcium concentration of the bile showed no significant difference among all the groups in Series Ⅰ, Ⅲ, and Ⅳ. Total bilirubin and each fraction showed no specific difference among them, either (Table 6).

DISCUSSION

In 1952, DAM and CHRISTENSEN[15] noticed the unexpected formation of gallstones in golden hamsters fed a fat-free diet. Using the method of alimentary production of gallstones in hamsters described by DAM et al., FORTNER (1954)[26], CAIRA et al. (1958)[8], and DREW (1963)[21] also observed gallstone formation in hamsters.

With the exception of DREWS, they all concentrated their attention on the vitamin imbalance ;

First, DAM et al.[9][10] investigated the effect of vitamins A, C, D, and E, inositol, folic acid, PABA, and choline on the formation of gallstones. None of these prevented the development of gallstones. But FORTNER noted that hamsters on vitamin A deficient diets developed gallstones more frequently than those receiving adequate amounts of vitamin A, and CAIRA et al. observed that the simultaneous deficiency of vitamins A, B, E, and K caused gallstones to develop in hamsters more frequently and rapidly. The animals used in these studies were fed sucrose as the carbohydrate component according to the early experiments by DAM et al. The occurrence of cholesterol gallstones is not uniform in hamsters fed a sucrose diet, as DAM himself stated after repeated experiments.

In our laboratory, up to this time, starch has been used as the main source of carbohydrate in the synthetic laboratory diet. Sucrose or glucose must be used for examining

the effects of pyridoxine and other vitamins upon EFA metabolism and/or gallstone formation, since vigorous multiplication of intestinal flora which might produce pyridoxine and various other vitamins is promoted by a starch diet. Moreover, EGUCHI[23] reported that the concentration of cholesterol in the liver of rats and hamsters fed glucose or sucrose is significantly higher than in those fed starch.

The occurrence of cholesterol gallstones was much less frequent in hamsters fed a fat-free starch diet than in those fed a fat-free sucrose diet, as indicated in Series I. Sucrose is hydrolysed in the intestinal canal to glucose and fructose. Therefore, it is necessary to use fructose or glucose as the sole source of carbohydrate. Cholesterol gallstones developed in some hamsters on sucrose or fructose diets but, only about half as many as in those on the glucose diet. In animals on glucose, sucrose and fructose fat-free diets the total bile acid concentration in the hepatic bile was lower than in those on the starch fat-free diet. These changes in total bile acids may be attributed to the following causes :

(1) When bile acids escape physiological destruction by the action of intestinal flora, the daily production of bile acids becomes lower as a result of the increase in the size of the hepatic pool.

(2) Blocking of the conversion of cholesterol to bile acids by certain enzyme systems, for example TPN-TPNH, and DPN-DPNH.

(3) Blocking of the conversion of cholesterol to bile acids by disturbance of EFA metabolism.

The half-life of C^{14}-cholic acid is shorter in starch-fed than in sucrose-fed rats, and the administration of cellulose as the indigestible residue makes the half-life of cholic acid the same as in those fed a chow diet[58]. It is lengthened by the administration of antibiotics[41]. The half-life and pool size of cholic acid are greater, and the daily production of bile acids lower[28] and the serum cholesterol level much higher[18] in germ-free than in normal rats. NATH et al[53]. and JOHANSSON et al[37]. reported that the most remarkable difference between the sucrose and the starch group in their intestinal flora was in the number of coli-form bacilli. However, mono-infection of germ-free rats with E. coli had no important influence on the half-life or daily production of cholic acid.[29]

The conversion of cholesterol to cholic acid in the liver requires DPN in the presence of AMP[2]. The metabolic pathway is, therefore, inhibited by a quantitative or functional deficiency of DPN, which might be brought about by high levels of TPN produced in the hexose-mono-phosphate shunt[13] in glycolysis in the liver.[57] Fructose is metabolized as fructose-6-phosphate not through this pathway, but a fructose diet produced cholesterol gallstones in hamsters in the present studies.

Thus, blocking of this conversion pathway may be provoked by disturbances of cholesterol activation. HIKASA et al[33] have thought that esters of cholesterol with arachidonic acid might be metabolized more rapidly than esters with saturated fatty acids. HIRANO[36] observed that pyridoxine deficiency supressed the esterification of cholesterol with arachidonic acid. WITTEN and HOLMAN[69] assumed that the conversion of linoleic acid to arachidonic acid was increased by the administration of pyridoxine. Large amounts of pyridoxine and biotin are usually produced by the intestinal flora[46], mainly coli-form. Since the ingested glucose, sucrose, or fructose can be absorbed completely in the upper

part of the intestine, an abnormal shift in intestinal flora (dysbacteria) may occur and result in pyridoxine deficiency[63].

The biliary cholesterol level and even, probably, the hepatic cholesterol level are greatly increased by glucose feeding. EGUCHI[23] observed that the hepatic cholesterol level was definitely increased in hamsters on a sucrose diet, but not in those on a starch diet. HIRANO[36] and VILLA et al.[68] reported that the hepatic cholesterol level was also increased in patients with cholesterol gallstones. These changes may be due to interference with the conversion of cholesterol to bile acids or to an increased synthesis of cholesterol in the liver. HANEL et al[30][31]. reported that no difference in the hepatic synthesis of cholesterol in hamsters was found between the lithogenic and non-lithogenic diet groups.

YOSHINAGA[72] and MARUYAMA[44] have stated that pyridoxine has an important influence on the hepatic synthesis of bile acids in rats. ALEKSANDROVA et al[1]. observed that the content of bile acids in the bile of rabbits was increased by the administration of pyridoxine. Therefore, it comes into question whether pyridoxine deficiency induce the gallstone formation.

No weight gain was observed in the starch pyridoxine-free diet group in 6 weeks after the start of the experimental feeding period, but acrodynia was not very evident and no gallstones were found (Group A in Series II). It is presumed that in the starch diet group, abundant pyridoxine was synthesized by the intestinal flora. MORGAN et al[48]. noted that signs referable to pyridoxine deficiency occurred only irregularly in lactose, dextrin and starch diet groups, but were constant and more severe in the sucrose diet group. MELLER et al. have succeeded in the development of vitamin B_6 deficiency in human subjects by using desoxypyridoxine. In the present studies, this antagonist was also given to hamsters in consideration of the presence of pyridoxine stored in the tissues (Group B in Series II). Even in these animals no gallstones developed. HIRANO[36] observed that cholesteryl arachidonate decreased rapidly in the liver of rats fed a pyridoxine-deficient diet supplemented with lard and treated with parenteral ACTH-Z. MARUYAMA[44] reported that daily intramuscular injections of ACTH-Z decreased the total bile acids in the liver of rats fed a pyridoxine-deficient diet. Under the same condition, however, the animals on a pyridoxine-deficient diet treated with ACTH-Z did not develop gallstones (Group C in Series II).

In Groups A, B, and C in Series II, an investigation was made of how severe pyridoxine deficiency develops animals on a starch diet. The formation of gallstones was observed in pyridoxine-deficient animals on a diet containing sucrose and animal fat (Group D in Series II). These gallstones, however, were brown and were found to contain traces of cholesterol when measured by LIEBERMANN-BURCHARD's reaction. Then indigestible ingredients, such as C. M. C. and agar-agar, which would favor the proliferation of intestinal flora, were added to the sucrose fat-free diet. In these groups the development of gallstones was markedly prevented and delayed, probably because of the alteration of the concentration of bile acids in the portal blood or the increased synthesis of pyridoxine by the intestinal flora. YANO[71] observed that the addition of cellulose to the diet increased the fecal and urinary excretion of pyridoxine.

Since the collection of hepatic bile in the groups in Series II was not achieved, the

relationship between pyridoxine deficiency and the behavior of bile acids in the bile remains uncertain. However, it appears definite that the intestinal flora is involved in the formation of gallstones, in some sense, although the development of gallstones is not due to pyridoxine deficiency alone.

For many years it has been thought that diets rich in animal fat are a major factor in the pathogenesis of gallstones[12)20)45)]. It was found by HIRANO[36)] that fatty acids esterified with cholesterol in the liver of patients with gallstones showed an EFA-deficient pattern. However, he indicated also that the linoleic acid level in the liver of these patients did not decrease in normal subjects. This may be attributed to the fact that in human beings taking fats from various food stuffs an absolute fat-free condition cannot be produced.

In Series III the sort of fatty acid most suitable for affecting directly or indirectly the mechanism of gallstone formation was investigated. Five sorts of fats were chosen for the following reasons (cf. Table 3) :

(1) Sesame oil as an EFA-rich fat, half of it consisting of linoleic acid.

(2) Lard as animal fat, containing greater amounts of longer-chain saturated fatty acids.

(3) Cod liver oil as a highly polyunsaturated fatty acid rich fat.

(4) Butter as a fat containing many short-chain fatty acids and the one used most commonly by Europeans.

(5) Coconut oil as a fat containing many more lower saturated fatty acids than butter.

Starch- and lactose- fat diet groups in Series III produced no gallstones, and the sucrose sesame oil diet group produced only pigmented stones. SHIODA[64)] observed that cholesterol gallstones appeared, but uncommonly, in the 10 % lard sucrose group and rarely in the 10 % sesame oil sucrose group. In the group fed a glucose diet with 10 % lard containg 2 % salt (Group G in Series III) cholesterol gallstones developed in 40 % of the animals, but in the cod liver oil glucose diet group no gallstones formed. The supplementation of the basal glucose diet with 10 % butter (Group D in Series III) produced many pure cholesterol gallstones. In the coconut oil diet group (Group F in Series III) they were also observed in almost all animals, and weight loss and skin lesions were also found as in the animals fed fat-free diets in Series I., because coconut oil contains only 1.5 % linoleic acid, which may not be enough (Table 5).

The levels of biliary cholesterol, total bile acids and lipid-p in bile in the butter group were very similar to those in the glucose fat-free group : i. e., high levels of cholesterol, low levels of total bile acids, phospholipid and ratio of total bile acids to cholesterol. This combination appears to facilicate the precipitation of cholesterol in bile.

Sesame oil contains 48.9 % linoleic acid : lard, 10.8 % : butter, 2.5 % : coconut oil, 1.5 % : cod liver oil, 4.2 %. Cod liver oil, however, contains many highly unsaurated fatty acids. KANEDA[38)] found that those in cod liver oil also have the physiological effects of EFA. Short-chain fatty acids are numerous in coconut and butter— a very different pattern from that of lard. Even when linoleic acid was added to the diet in doses of about 200 mg/day in the form of butter fat (a sufficient amount of EFA for normal

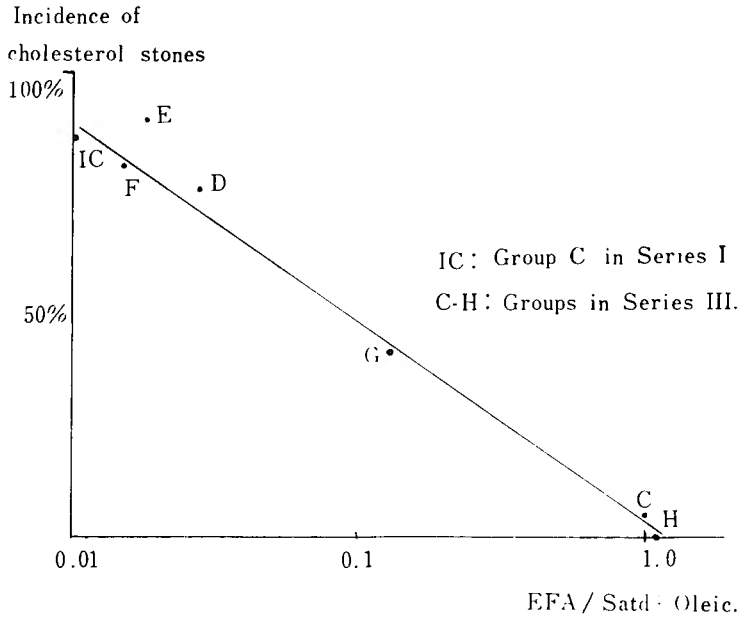

Fig. 1 The Relationship between EFA in the Dietary Fats and the Incidence of Cholesterol Stones in Hamsters.

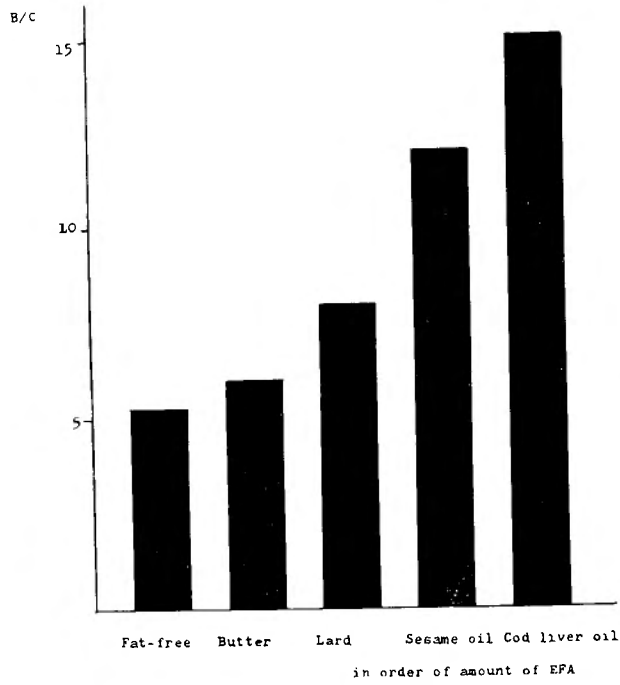

Fig. 2. The Relationship between EFA in Dietary Fats and the Ratio of Total Bile Acids to Cholesterol in the Bile of Hamsters.

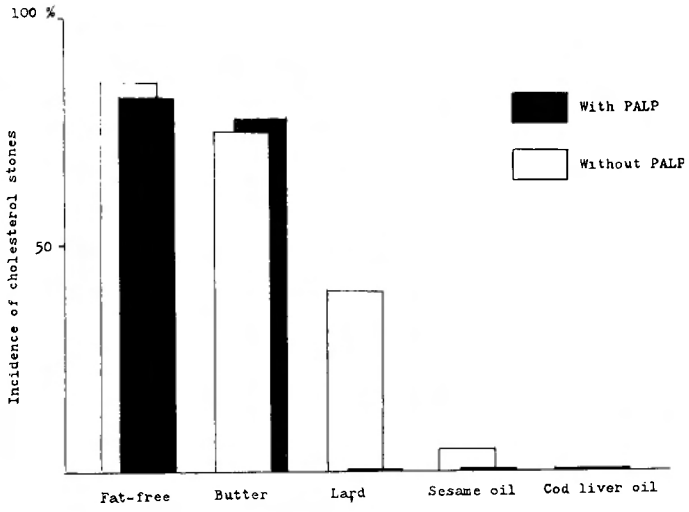

Fig. 3. Effect of PALP on Cholesterol Stone Formation in Hamsters.

growth of animals), it could not prevent cholesterol gallstone formation. It is evident in Series III that the formation of cholesterol gallstones may be induced by a decrease of cholesterol catabolism due to disturbances of EFA metabolism in vivo, which might be brought about indirectly by the high content of short-chain fatty acids in butter. Therefore, a relationship was found between the EFA content and the incidence of cholesterol gallstones ; that is, the higher the ratio of EFA to saturated fatty acids plus oleic acid, the lower was the incidence of cholesterol gallstones (Fig. 1).

FITZGERALD[25] found no difference among fat-free, corn oil, and coconut oil diets with respect to the multiplication of intestinal flora in rats fed corn starch. NATH et al.,[53] however, demonstrated that butter decreased the intestinal flora more than corn oil in rats on a sucrose diet.

Though HOLMAN's hypothesis that pyridoxine increases the conversion of linoleic acid to arachidonic acid was opposed by KIRSCHMAN et al[42]., NAKAMURA et al[52]. observed an increase of arachidonic acid in the serum after the administration of PALP, but not of pyridoxine. WAKIL[73] also observed that PALP increased the rate of incorporation of acetyl CoA in long-chain fatty acids, whereas pyridoxine did not. For these reasons, PALP (20 times the amount in the ordinary diet) was injected daily in the four fat diet groups in Series IV.

PALP injections did not influence the formation of cholesterol gallstones in the butter group, but in the lard and sesame oil groups they completely prevented the development of gallstones. The cod liver oil group treated with PALP had several pigmented stones. In the glucose fat-free diet group as many cholesterol gallstones developed in those treated with PALP as in those not receiving PALP (Table 5 and Fig. 3). These findings suggest that the fatty acids compete for esterification with cholesterol. In the butter group, PALP could not prevent the occurrence of cholesterol gallstones even with adequate supplementation with linoleic acid, although it did protect both the sesame oil and lard groups.

PALP injections increased the concentrations of total bile acids and phospholipids in the bile in each group in Series IV Among them, the alteration of bile acids was most remarkable (Table 6). The ratio of total bile acids to cholesterol rose as the amount of EFA increased (Fig. 2). Therefore, above two findings in Fig. 1 and 2 indicate that metabolic disturbances of hepatic cholesterol with quantitative and qualitative deficiency of EFA induce the low ratio of total bile acids to cholesterol in the bile and facilicate the formation of cholesterol gallstones.

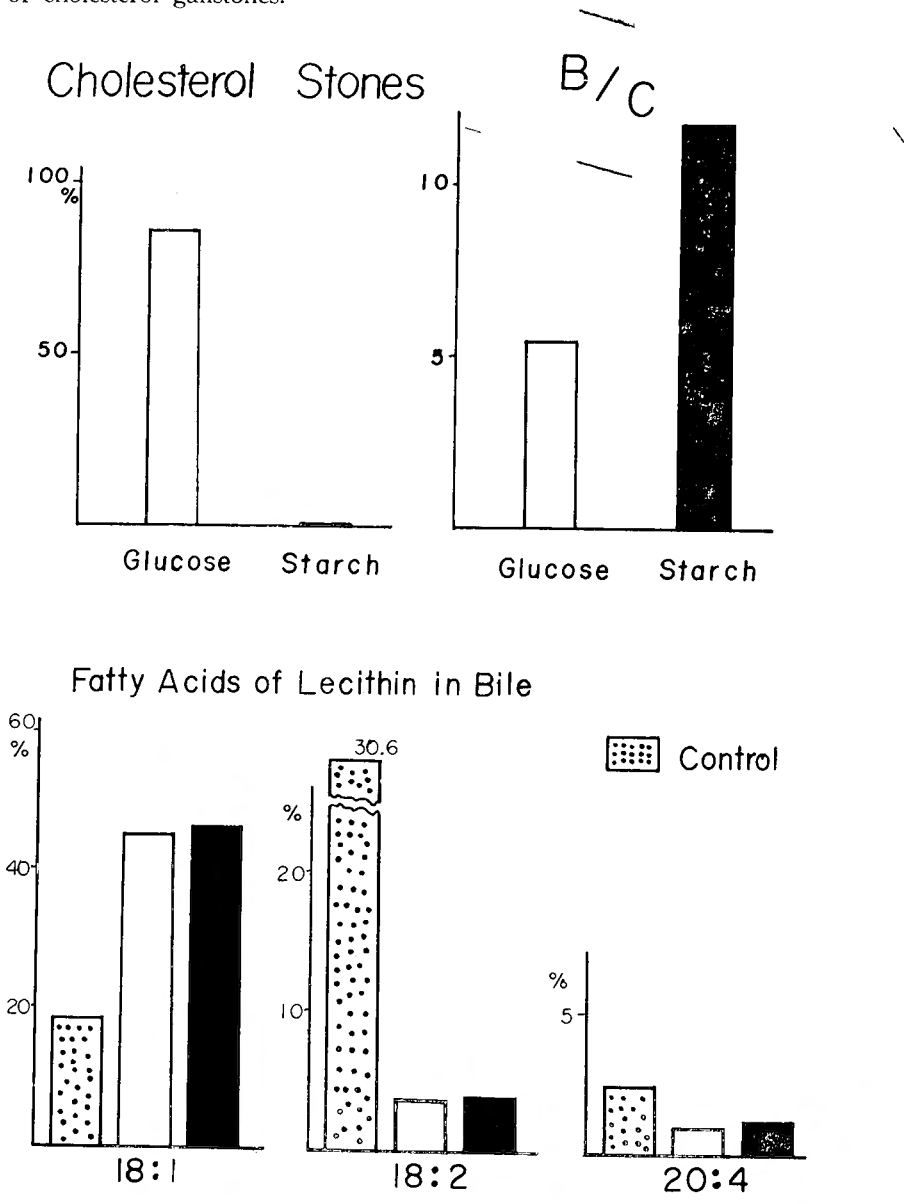

Fig. 4 Difference of Effects on the Gallstone Formation and the Bile Constituents between Glucose - and Starch Diet Group.

MIYAKE et al[47]. emphasized that the most important factor in gallstone formation was the alteration of the fatty acid components of lecithin in the bile, since animals on a fat-free diet had less linoleic and arachidonic acids and more oleic acid in their bile than those fed sunflower seed oil. However, the fatty acid pattern in the bile of hamsters in the glucose fat-free and starch fat-free diet group in Series I was the same, although cholesterol gallstones appeared in the former group. Moreover, the administration of purified linoleic acid as the source of EFA and the simultaneous parenteral injection of PALP in the glucose diet group caused a marked increase in linoleic acid (68.7%) and a decrease in oleic acid (5.8 %) in the bile. Arachidonic acid in the bile did not increase markedly. The fatty acid composition in the bile of hamsters fed cod liver oil, which contains a small amount of linoleic acid and many highly unsaturated fatty acids, showed a pattern similar to that of cod liver oil itself. However, the pattern of fatty acids in the bile of the butter group was indeed very similar to that of those in the fat-free groups, and the pattern of fatty acids in the lard group was intermediate between the butter and sesame oil groups (Table 7). In a word, although the composition of fatty acids in bile is greatly affected by variations of dietary fats, it is evident that another more important factor may exist.

SUMMARY AND CONCLUSION

Young golden hamsters were fed various diets for 4–8 weeks. The relationship of several nutrients, such as carbohydrates, fats, and vitamin B_6, to the incidence of experimental gallstones in hamsters was invetigated.

(1) Cholesterol gallstones were produced with great regularity in the glucose fat-free diet group. In the sucrose or fructose diet groups, the incidence of cholesterol gallstones was about half as great. The starch fat-free diet did not produce gallstones.

(2) The simultaneous administration of ACTH-Z or desoxypyridoxine to the starch diet group had no effect on the formation of gallstones. The addition of C. M. C. or agar-agar to the sucrose fat-free diet decreased and delayed the development of cholesterol stones.

(3) The coconut oil- and the butter- glucose diet induced many cholesterol gallstones, but in the lard- and the sesame oil- glucose diet groups their formation was completely prevented by the simultaneous injection of PALP. A relationship was found between the EFA content in the dietary fats and the incidence of cholesterol gallstones ; that is, the higher the ratio of EFA to saturated fatty acids plus oleic acid, the lower was the incidence of cholesterol gallstones.

(4) Pigmented hard stones, which sometimes appeared in the fat-free sucrose and in the sesame oil- or cod liver oil- glucose diet groups, were found to consist of bile salts and calcium phosphate by chemical and infrared spectral analyses.

(5) The analysis of hepatic bile showed that the biliary cholesterol level increased and the total bile acid concentration decreased, and that the ratio of total bile acids to cholesterol decreased in the glucose fat-free, sucrose fat-free, fructose fat-free, and glucose-butter diet groups. This combination appears to facilicate the formation of cholesterol gallstones. The ratio of total bile acids to cholesterol rose as the amount of EFA increased

in the dietary fats.

(6) The fatty acid composition of the bile in animals with cholesterol gallstones showed an EFA-deficient pattern ; viz., a decrease of linoleic and arachidonic acids, and an increase of oleic and trienoic acids. However, these results were also obtained in the starch fat-free diet group, in which gallstones were not produced.

(7) Absolute EFA deficiency was not observed even in patients with cholesterol gallstones. Therefore, when a great amount of short-chain fatty acids is taken, disturbance of EFA and cholesterol metabolism in vivo might be produced and cholesterol gallstones might be formed. Thus, it is to be expected that cholesterol gallstones form more frequently in Europeans and Americans, who take more butter and other animal fats containing a greater amount of short-chain fatty acids.

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Fig. 5. Cholesterol gallstones in the glucose fat-free diet group. (×16)

Fig. 6. Amorphous pigmented stones in the sucrose sesame oil diet group. (×16)

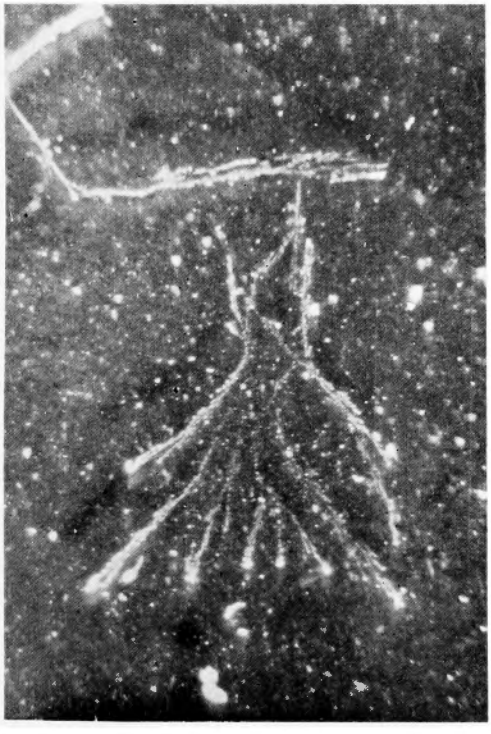

Fig. 7. Cholesterol crystals in the dried bladder bile of hamsters with cholesterol gallstones. (×16)

Fig. 8. Cholesterol gallstones in the glucose butter diet group. (×16)

和文抄録

胆石の成因に関する実験的研究

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胆石症が日常の臨床に於て屢々認められる疾患であるにも拘らず，その成因は依然として不明であるといえる．近年，胆石の発生が地域的人種的特殊性を有する事から食餌による栄養素の代謝機序との関連性に於て動物性脂質摂取が胆石殊にコレステロール系結石（「コ」系石）の成因の一つではないかと漠然と想像されては来たが，未だ実験的に証明されたものではない．

我々の教室では，日笠等のラッテに於て不可欠脂酸（EFA）及び Vit. B_6 が肝に於けるコレステロールから胆汁酸合成過程及び胆汁中への胆汁酸排泄に重要なる意義を有することを証明した．更に塩田及び Dam 等はハムスターに於て無脂肪飼育時のみ「コ」系石を生ずる事を認めた．併しながら平野による胆石患者の肝の脂酸構成の分析からも人体に於てリノール酸等 EFA の絶対的欠乏はあり得ないと考えられる．従つてEFA は投与されていながらも胆石が尚且つ発生する状態は如何なるものであるかが究明されねばならない．斯る関点より，ハムスターを絶対的 EFA 欠乏群，Vit. B_6 欠乏 EFA 投与群，各種脂肪投与群及び Vit. B_6 の活性型としてピリドキサール燐酸（PALP）併用群に分けて一定期間各々の合成飼料で飼育し，実験的胆石形成と EFA 及び Vit. B_6 との関連性を追求すると共に，それ等より得た肝胆汁の分析を行なつてその中の最も重要なる因子の解明を試み，次の如き結果を得た．

(1)　「コ」系石はブドウ糖 EFA 欠乏群に於て高率に発生を認めた．蔗糖及び果糖 EFA 欠乏群はその約半数に於て同様「コ」系石を生じた．併し澱粉 EFA 欠乏群は胆石の発生を認めなかつた．

(2)　澱粉 Vit. B_6 欠乏群に於ける ACTH-Z 或いはデゾキシピリドキシン投与は何ら結石をもたらさなかつた．併し蔗糖 EFA 欠乏群に於ける多量のセルロースや寒天の添加は「コ」系石の発生を抑制且つ遅延させた．

(3)　ブドウ糖群に於けるヤシ油及びバターの投与は高率に「コ」系石を生じた．ラード投与は低率に，ゴマ油投与は極く稀に「コ」系石発生を認めた．従つて「コ」系石の発生は食餌中の脂肪に於ける EFA の飽和脂酸に対する比率の小なるほど発生し易くなることが見出された．且つラード及びゴマ油投与に PALP の併用は完全に「コ」系石を抑制した．

(4)　硬い色素石が蔗糖 EFA 欠乏群，ブドウ糖ゴマ油又は肝油群に於て出現したが，それ等は胆汁酸塩及び燐酸カルシウムを多量に含み胆汁色素は極く少量であり，「コ」系石とは明かに区別されると共に人体に於ける所謂色素石とも一致しないと考えられた．

(5)　胆汁中コレステロール量（C）の増加，総胆汁酸量（B）の減少，それ等の比（B/C）の低下がブドウ糖，蔗糖，果糖の各 EFA 欠乏群及びブドウ糖バター群で認められた．更にこの B/C は EFA の量に比例して高値を呈する事を見出した．従つてこれ等の変化が「コ」系石の発生しやすい状態を作るものと考えられる．

(6)　「コ」系石を生じた動物の胆汁の脂酸構成は EFA 欠乏型を呈したが，この欠乏型は「コ」系石の発生を認めなかつた澱粉 EFA 欠乏群に於ても同様に認めた．従つて胆汁脂酸構成の変化のみが胆石形成の主要因子とは考え難い．

(7)　「コ」系石を有する胆石患者に於ても絶対的 EFA 欠乏状態は観察されなかつた事と以上の実験成績を併せ考えると，多量の低級脂酸が摂取された時，生体に於ける EFA 代謝障害ひいてはコレステロールの代謝障害が惹起され，「コ」系石が発生して来ると考えられる．更にこの事は低級脂酸を多量に含有するバター等の動物脂質を多く摂取している欧米人に於て「コ」系石が多数認められる事とよく一致するものと思われる．